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Low genetic diversity in Nurseryfish, *Kurtus gulliveri* (Perciformes: Kurtidae), and an appraisal of its breeding system using microsatellite loci

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ABSTRACT

The Nurseryfish, *Kurtus gulliveri*, is a freshwater fish distributed in northern Australia and southern New Guinea that exhibits forehead brooding, a unique form of male parental care. Microsatellite markers were developed for paternity analysis to study its reproductive life history in a population from the Adelaide River, Northern Territory, Australia. In 20 microsatellite loci tested, only two polymorphic markers were found. The microsatellite data are mostly consistent with the hypotheses that the putative egg-carrying male sired the egg mass and no multiple paternity occurred in the population examined. However, caution should be taken in interpreting the results, given the low genetic diversity and limited analytical power. The extremely low genetic diversity was also corroborated by mitochondrial DNA, the most variable fragment in the piscine mitogenome. Eighteen individuals from the Adelaide River were sequenced, revealing four polymorphic sites in the control region and five haplotypes in total, with an average p-distance of 0.001. Additional individuals from three other isolated populations in the Northern Territory (Daly River, South Alligator River and East Alligator River) were sequenced and found to be identical to one of the common haplotypes from the Adelaide River. Four specimens collected from New Guinea represent a single haplotype that had 24.5 substitutions, on average, relative to the Australian populations.

KEYWORDS: Nurseryfish, *Kurtus gulliveri,* low genetic diversity, microsatellites, control region, breeding system.

INTRODUCTION

The Nurseryfish, *Kurtus gulliveri* Castelnau, 1878, is known for its unusual method of parental care. Weber (1910, 1913) and Beaufort (1914) reported that the male carries the egg mass on a supraoccipital hook, a method termed forehead brooding by Balon (1975). Virtually nothing had been published on the biology of this unique species in the subsequent 90 years until a life history study was begun in 2001 at the Adelaide River near Darwin in the Northern Territory of Australia (Berra 2003).

Kurtus gulliveri is known from northern Australia and southern New Guinea (Fig. 1). It is distinct from the only other member of the family Kurtidae, *K. indicus* Bloch, 1786, which occurs from India to Borneo (Berra 2003, 2007). Recent studies of functional morphology and population assessments (Berra 2003; Carpenter *et al.* 2004; Berra *et al.* 2007) provide a basis to explore the unique spawning habits of *K. gulliveri*. The anatomy and histology of the male's supraoccipital hook has been examined, and Berra & Humphrey (2002) speculated that engorgement with blood in the highly vascularised dermis of the hook may help hold egg masses in place. This species feeds on arthropods and teleosts (Berra & Wedd 2001), has been documented to live a maximum of four years (Berra & Aday 2004), and has a significantly skewed sex ratio of 65 percent male to 35 percent female (Berra *et al.* 2007), although skew might be caused by the male's hook increasing vulnerability to gill netting. It possesses very small chromosomes with a karyotype 2n = 44 (Ezaz *et al.* 2006).

Recent investigations in fish reproductive behaviours have included genetic analysis of parentage with molecular markers. In 21 percent of all teleost families, there is parental care of offspring; of those families, 70 percent of the species' parental care is provided entirely by the male (Avise *et al.* 2002). Although male parental care is common, the novel mode of care in Nurseryfish elicits many questions about its reproductive behaviour. Is the egg mass carried by a male the product of a single female? Conversely, is the egg-carrying male the only sire, or is there competition among males to fertilise egg masses before or after lodging them on their forehead, thus resulting in multiple paternity? Does this mode of reproduction yield any aberrant patterns in terms of population-genetic structure? Instances of multiple paternities are unknown, as molecular data have never been collected in the species. At the most basic level, how genetically diverse is *K. gulliveri* within a river and among different rivers? Considering the difficulty of observing behaviours in nature and the paucity of information on the genetic diversity and reproductive natural history of this obscure species, microsatellite loci were developed and the mitochondrial control region was sequenced in order to: 1) survey genetic diversity in the species, 2) determine whether egg-carrying males sire the egg mass they carry, and 3) test for multiple paternity in egg masses. Multiple paternity and alternative life history strategies are well documented in fishes (Breder & Rosen 1966; reviewed in Avise *et al.* 2002), and microsatellite paternity analysis is a widely used tool to test hypotheses about these behaviours (Bruford *et al.* 1996). If *K. gulliveri* uses alternative reproductive tactics such as sneaking (Avise *et al.* 2003) or satellite fertilisations (Neff 2001), multiple paternity should be detectable in the eggs masses.

MATERIALS AND METHODS

Collection sites and materials examined. The majority of specimens utilised for this study were collected by gill netting in Marrakai Creek (12°40.950'S, 131°20.030'E), a major freshwater tributary of the Adelaide River about 65 km east of Darwin (Fig. 1). Three additional samples provided by the Museum and Art Gallery of the Northern Territory from other isolated populations in northern Australia (Daly River, South Alligator River and East Alligator River) and four specimens from New Guinea were acquired from the Kansas University Biodiversity Institute tissue collection for comparison with the Adelaide River population (Fig. 1). Tissue samples of adults and egg masses were preserved in 98 percent ethanol immediately upon capture. Collections were made in 2001, 2003, 2004 and 2009 during the dry season between late June and early November, coinciding with Nurseryfish spawning season (Berra & Neira 2003; Berra *et al.* 2007).

Forty-one adults from the Adelaide River (Table 1) were used to estimate allelic frequencies and other parameters for the microsatellite loci. Seven egg masses (EM-01 to EM-07) in varying stages of development and colouration caught at varying distances from the most proximate male

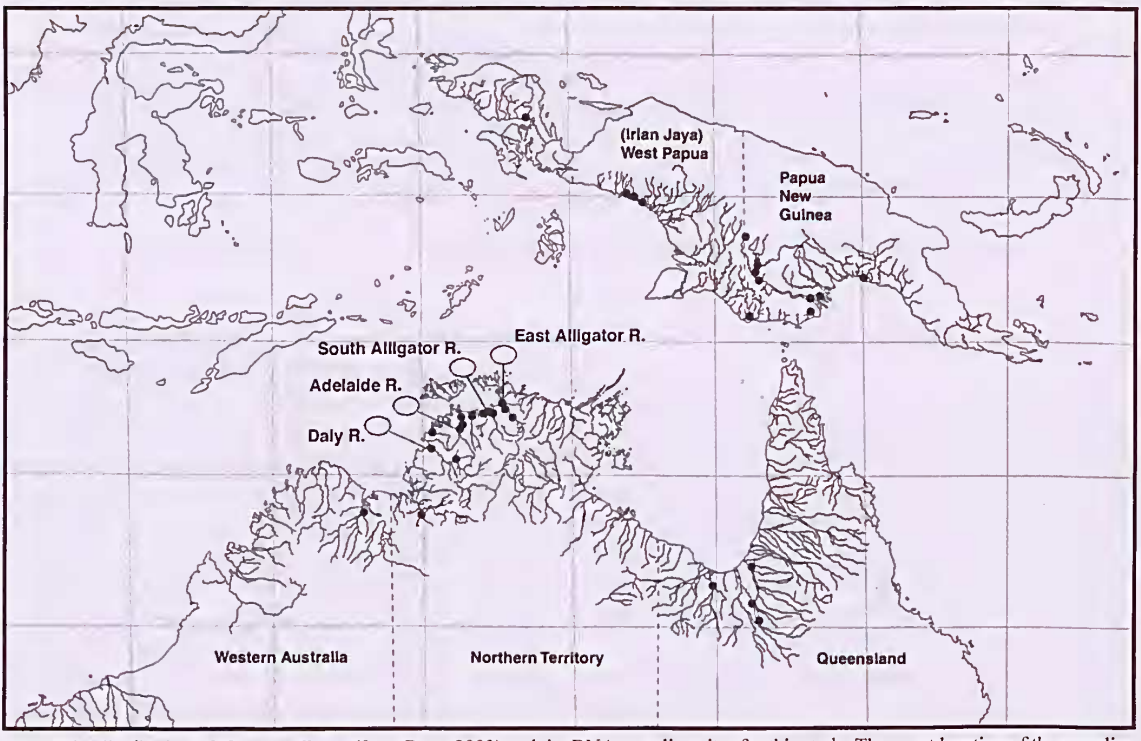

Fig. 1. The distribution of *Kurtus gulliveri* (from Berra 2003) and the DNA sampling sites for this study. The exact location of the sampling site in New Guinea is unknown.

Table 1. Description of specimens used in this study. All samples from Marrakai Creek were collected between 2001 and 2004. Lab codes denote adults (AD), including males associated with egg masses. EM-01 to EM-06 correspond to masses associated with adult males with matching numbers (EM-07 is not an associated mass but was tested for association to AD-06 male found 60 cm away in net). EM-08 to EM-14 are unassociated masses. U = sex unknown. Pink = fertilised.

Lab code	Sex	Tissue type	Description	Berra field code	Year
AD-01	male	adult	Associated w/ mass	TMB01-14	2001
EM-01		egg mass	Pink mass assoc. w/ male	TMB01-14	2001
AD-02	male	adult	Associated w/ mass	TMB04-7#2	2004
EM-02		egg mass	Pink mass assoc. w/ male	TMB04-7#2	2004
AD-03	male	adult	Associated w/ mass	TMB03-9	2003
EM-03		egg mass	White mass w/ assoc. male	TMB03-9#1	2003
AD-04	male	adult	Associated w/ mass	TMB04-6 #4	2004
EM-04		egg mass	Pink mass assoc. w/ male	TMB04-6#3	2004
AD-05	male	adult	Associated w/ mass	TMB04-7 #1	2004
EM-05		egg mass	Pink mass assoc. w/ male	TMB04-7#1	2004
AD-06	male	adult	Associated w/ mass	TMB04-6	2004
EM-06		egg mass	Pink mass assoc. w/ male	TMB04-6#1	2004
EM-07		egg mass	Pink mass assoc. w/ male	TMB04-6#2	2004
EM-08		egg mass	White. No assoc. male. Fertilised	TMB03-15	2003
EM-09		egg mass	White. No assoc. male. Fertilised	TMB04-3#2	2004
EM-10		egg mass	Pink. No assoc. male	TMB04-5	2004
EM-11		egg mass	Pink. No assoc. male	TMB03-9#2	2003
EM-12		egg mass	Pink. No assoc. male	TMB01-27#1	2001
EM-13		egg mass	White. No assoc. male. Did not amplify	TMB03-9#3	2003
EM-14		egg mass	White. No assoc. male. Did not amplify	TMB03-8	2003
AD-07	male	adult	screening adult	TMB01-10	2001
AD-08	female	adult	screening adult	TMB01-10	2001
AD-09	female	adult	screening adult	TMB01-10	2001
AD-10	female	adult	screening adult	TMB01-10	2001
AD-11	female	adult	screening adult	TMB01-10	2001
AD-12	male	adult	screening adult	TMB01-11	2001
AD-13	female	adult	screening adult	TMB01-11	2001
AD-14	female	adult	screening adult	TMB01-11	2001
AD-15	female	adult	screening adult	TMB01-11	2001
AD-16	female	adult	screening adult	TMB01-11	2001
AD-17	female	adult	screening adult	TMB01-10	2001
AD-18	U	adult	screening adult	TMB01-10	2001
AD-19	male	adult	screening adult	TMB01-10	2001
AD-20	U	adult	screening adult	TMB01-8	2001
AD-21	female	adult	screening adult	TMB01-10	2001
AD-22	male	adult	screening adult	TMB01-10	2001
AD-23	U	adult	screening adult	TMB01-6 #2	2001
AD-24	U	adult	screening adult	TMB01-4 #9	2001
AD-25	U	adult	screening adult	TMB01-4 #6	2001
AD-26	U	adult	screening adult	TMB01-4 #7	2001
AD-27	U	adult	screening adult	TMB01-4 #1	2001
AD-28	U	adult	screening adult	TMB01-4 #3	2001
AD-29	U	adult	screening adult	TMB01-4 #2	2001
AD-30	U	adult	screening adult	TMB01-4 #4	2001
AD-31	U	adult	screening adult	TMB01-4 #5	2001
AD-32	U	adult	screening adult	TMB01-4 #8	2001
AD-33	female	adult	screening adult	TMB01-10	2001
AD-34	female	adult	screening adult	TMB01-10	2001
AD-35	female	adult	screening adult	TMB01-10	2001
AD-36	U	adult	screening adult	TMB01-15	2001
AD-37	female	adult	screening adult	TMB01-10	2001
AD-38	female	adult	screening adult	TMB01-10	2001
AD-39	U	adult	screening adult	TMB01-6 #1	2001
AD-40	female	adult	screening adult	TMB01-10	2001
AD-41	female	adult	screening adult	TMB01-10	2001

in the net were analysed (see Fig. 2 and Table 1 for field codes and sample descriptions). In cases where the egg mass was lying next to the male in the gill net, it presumably dislodged when the male struck the net, which has been observed sometimes in the field (Berra *et al.* 2004). Our working hypothesis is that males collected either touching or in close proximity (<5 cm) to an egg mass, e.g., male AD-06 (Fig. 2A) and AD-05 (Fig. 2B), carried the egg mass on their supraoccipital hook as they struck the net and sired any embryos they carried (EM-06 and EM-05, respectively). The extreme difficulty of catching males with an intact egg mass is described by Berra *et al.* (2004). Three additional pink egg masses with eyed embryos trapped in the net without a male nearby (EM-10, EM-11, and EM-12) and four white egg masses that did not show any obvious signs of development (EM-08, EM-09, EM-13 and EM-14) were also analysed.

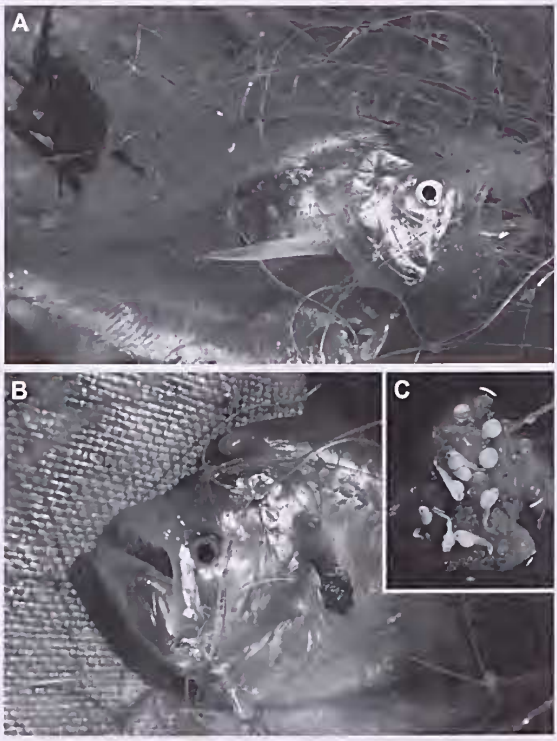

Fig. 2A. Male Nurseryfish (AD-06) (210 mm SL) with detached egg mass caught and photographed in gill net on 11 August 2004 (EM-06). Note the edematous, supraoccipital hook and the proximity of the foreground egg mass. This male was initially presumed to be carrying the foreground egg mass that detached upon contact with the gill net. Microsatellite data from one locus (Kg019) indicate the embryos in the foreground egg mass (EM-06) are consistent with AD-06 as the father, but the background egg mass (EM-07) is not consistent with this male as the father (Table 4). However, locus Kg275 data show that the embryos in the foreground (EM-06) and background egg mass (EM-07) could not have been fathered by this male; **B,** Male Nurseryfish (AD-05) (285 mm SL) with immediately adjacent detached egg mass fragment (EM-05) collected and photographed on 13 August 2004 (EM-05); **C,** (inset) Close up of reverse side of egg mass fragment from Fig. 2B showing eyed embryos and yolk sac fry approx. 5.5 mm SL. The alleles of these fry are consistent with fertilisation by male AD-05 in Fig. 2B that was presumably carrying the egg mass.

The total number of individual eggs or embryos per egg mass varied from $n = 8$ in a small (partial) egg mass with embryos to $n = 1300$ in a presumably complete egg mass (Berra 2003; Berra *et al.* 2007).

DNA extraction. DNA was extracted from muscle and eggs using Qia-quick DNA extraction columns (Qiagen Inc., Valencia, California). From each egg mass, 8–65 embryos or eggs were extracted contingent upon the total number found in the mass. Specifically, all embryos of the mass were extracted if the mass consisted of 20 eggs or less. Conversely, if the mass consisted of more than 1000 embryos, 65 embryos were randomly chosen then extracted via a Chelex 100 method (Walsh *et al.* 1991).

Microsatellite marker development. Total genomic DNA was extracted from a single adult (AD-12), and microsatellite loci were isolated using the method described by An *et al.* (2004). Isolation of targeted genomic fragments, the method of enrichment-capture with stringency washes, and buffer composition followed Hammond *et al.* (1998). A total of 475 colonies containing inserts was amplified and sequenced in one direction using BigDye Terminator chemistry (Applied Biosystems) and M13F primer to screen for microsatellite inserts. Forty-six positive clones (that contained microsatellites) were sequenced in the opposite direction with M13R. Complementary forward and reverse sequences were assembled using Sequencher 3.0 (Gene Codes Corp.). Twenty-four of the 46 possessed adequate flanking regions and a repeat section >12 base pairs. PCR conditions for the newly designed primers were optimised using a temperature gradient with annealing temperatures ranging from 48–62°C to determine optimum amplification conditions. Each reaction contained the following: 2.0 µl 1mM dNTPs, 1 µl of 10x PCR buffer, 0.4 µl of 50mM $MgCl_2$, 0.4 µl 10mM F primer, 0.4 µl 10mM R primer, 0.1 µl (0.5U) Taq polymerase (Invitrogen), 4.7 µl ddH_2O and 1.0 µl (50ng) genomic DNA. Thermo-cycling conditions used an initial denaturation step at 94°C for 2 minutes followed by 30 cycles of 94°C (30 seconds), optimal annealing temperature (30 seconds), 72°C (60 seconds) followed by a final extension at 72°C for 10 minutes. Of the 24 primer-pairs tested, only three failed to amplify a product of the predicted size and one was a duplicate microsatellite. Sequences of the 20 loci were deposited in GenBank (Table 2). Genotypic data were collected for each locus in all 41 adults from the Adelaide River. Products were visualised with an ABI PRISM 310 Genetic Fragment Analyzer (Applied Biosystems).

Marker screening and parentage analyses. Berra & Neira (2003) described egg masses as being individually attached to a fibrous raceme (Fig. 3), as originally described by Guitel (1913). Therefore, we made the assumption that each intact mass originates from a single female, as observed in the closely related apogonids (Vagelli 1999). To test this assumption, we attempted to extract DNA and amplify the control region (D-loop) from 'maternal' fibrous material (Fig. 3) holding the eggs together, but the results were

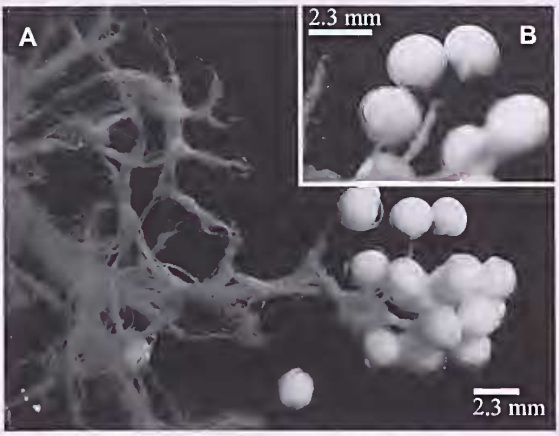

Fig. 3. Digital photomicrograph of partially dissected egg mass (EM-06): **A**, fibrous matrix of structures connecting each egg or embryo. Magnification approx. 8.7x; **B**, (inset) detail of fibre connections to each embryo in this fertilised mass. Magnification approx. 12x.

negative. Most likely, the fibrous material contained no DNA and our assumption remains tentative but reasonable.

Parental genotype reconstruction was performed with the program Gerud 2.0 (Jones 2005), which uses an algorithm to describe a minimum number of parental genotypes contributing to an array of typed offspring. This approach is based on Mendelian rules of inheritance but assumes that parental genotypes are unknown. In addition, Gerud 2.0 calculates exclusion probabilities for this 'neither parent known' model. The assumption of a single dam meets the criteria of the program Gerud 2.0 to accurately reconstruct parental genotypes by assuring that the progeny are full or half sibs. In addition, for those egg masses with net-associated males, we confirmed paternity by matching genotypes in the mass and in the adjacent male. We tested how powerful our microsatellite markers were at detecting multiple paternity by using PrDM (Probability to Detect Multiple matings) (Neff & Pitcher 2002). Following similar simulations used in Chapman *et al.* (2004), several mating scenarios were run with: 1) two males with equal mating success contributing to the mass; 2) two males with skewed mating success to simulate cuckoldry or sneaker fertilisations (66.7 percent and 33.3 percent); 3) three males with equal success contributing to the mass; and 4) three males with a skewed ratio (60 percent, 25 percent and 15 percent) of mating success. Several brood sizes were tested due to the variability in the sampled egg masses.

Mitochondrial DNA analysis. To further assess genetic diversity, the mtDNA Dloop was partially sequenced (780 bp) in 17 adults and one larva collected from the Adelaide River, three individuals from the Daly River, South Alligator River and East Alligator River (from the Northern Territory, Australia), and four individuals from New Guinea. Amplification conditions followed Sivasundar *et al.* (2001). Cycle sequencing reactions were performed with BigDye Terminator sequencing reaction kit (Applied Biosystems) and products were visualised with a MJ Research 5100 Basestation sequencer (MJ research, CA). Complementary sequences were edited with Sequencher 3.0 (Gene Codes Corp.) and aligned with Bioedit (Hall 1999) using Clustal W (Thompson *et al.* 1997). All mtDNA haplotypes were deposited in Genbank (EU262420–EU262436).

Mitochondrial Dloop data were used to estimate relevant demographic parameters for the Adelaide River population. The average p-distance among individuals was calculated using MEGA 3.1 (Kumar *et al.* 2004) and statistical parsimony (SP) networks were constructed using TCS 1.21 (Clement *et al.* 2000). Subsequently, the software package LAMARC (Kuhner & Smith 2007) was used to estimate historical demographic parameters (θ and g) under a Bayesian approach. Simulations were performed under a coalescent model with exponential growth to estimate the population parameters *g* (the exponential growth rate) and θ; $\theta=2N_f\mu$, where N_f is effective population size for females and μ is the mutation rate. MCMC chains were run with the following settings: one initial chain with 500 trees sampled, sampling increment = 20, trees discarded = 1000; one final chain with 10000 trees sampled, sampling increment = 100, trees discarded = 1000. Three independent runs (with different speeds) were performed and the results were consistent. A molecular clock of Dloop calculated for trans-isthmian geminate species of *Chromis* damselfishes (8.24 x 10-8 to 9.30 x 10-8 substitutions per site per generation, Domingues *et al.* 2005) was used to calibrate the effective number of females in the population.

RESULTS

Microsatellite loci. Genetic assays reveal that variation is unexpectedly low in this population: among 20 microsatellite loci screened for variation (n = 41 adult individuals) only two loci were polymorphic (Table 2). To confirm that there was no non-homologous amplification, all monomorphic loci were bidirectionally sequenced to confirm the presence of a microsatellite. The two polymorphic loci (Kg019 and Kg275) are suitable for paternity analyses, exhibiting 6 and 5 alleles respectively (Table 3). Neither locus deviated from the Hardy-Weinberg Equilibrium (HWE) (Kg019 p = 0.34, and Kg275 p = 0.48) as calculated in GENEPOP (Raymond & Rousset 1995). Single-locus exclusion probabilities (i.e., the probability of excluding a random individual from the population as a potential father) were low, at 25 percent and 26 percent per locus under the 'no parents known' model, with combined probabilities for both loci increasing to 44 percent (Table 3).

Tests of genotypic linkage disequilibrium were performed in Arlequin v3.0 (Excoffier *et al.* 2007) with 10,000 permutations using the EM algorithm. No linkage disequilibrium was detected (Chi-squared test value = 21.086, p = 0.687).

We sought to determine whether a male found in close proximity to an egg mass in the net during capture could have sired and carried the eggs. Five males and five

Table 2. Name and Genbank accession number of 20 microsatellite loci for *Kurtus gulliveri* developed for this study. PCR primer sequences (bp) including priming sites and optimal annealing temperature (T) are given for each locus. The number of distinct alleles (*k*), observed and expected heterozygosities, and Hardy-Weinberg Equilibrium test results (H_O, H_E, and HWE) are given for the only two polymorphic loci found in a sample of 41 adult Nurseryfish.

Locus-Accession no.	Primer Sequence (5'-3')	Sequence motif	T	Size (bp)	*k*	H_O	H_E	HWE
Kg004 EF439631	F:TGAGCTCCAGACAGATGTC R:TGAAGTTCCACTTTGGAGACG	$(CCT)_9$	58.4	302	1	-	-	-
Kg007 EF439632	F:CAGGGCACAGTTTGCATTTTGT R:CACCCTGCTCGGAGTGACCTTATT	$(GT)_{19}$	58.6	118	1	-	-	-
Kg009 EF439633	F:CTAACAGCAGGGCAGAGAGC R:CACTGGTAACGCTGCAGACC	$(GT)_{16}(GC)_4(GT)_{20}$	57.4	243	1	-	-	-
Kg010 EU262437	F:GGTGTGTGCATGTGGGTTTA R:GGGCCAAAATGTGTAAATGG	$(CT)_{12}(GT)(CT)_3$	58.6	401	1	-	-	-
Kg012 EF439650	F:GAATGAAGGGCGAGAGAGTG R:CTGAGTGTTTTTGCGTTTGC	$(GA)_{17}$	60.5	306	1	-	-	-
Kg019 EF439634	F:CGGATTTATTTGGCCTGTGA R:GCTCCTGGATCAACGTAAGC	$(CA)_{24}$	58.6	222	6	0.61	0.68	p= 0.3395
Kg026 EF439635	F:AGGGCAGGATTTTCAGTGTG R:CACCAGAAACAAAGGACACG	$(GT)_{24}$	60.5	281	1	-	-	-
Kg030 EF439636	F:AAGCTTGGGATCGTGAGATG R:ACCGGCTGTGTGTTTGAAG	(GA)n(CA)n Imperfect 104 bps	58.4	195	1	-	-	-
Kg034 EF439637	F:CCGTTTCTTGGAAGGTTGAC R:ATTCTCGCCAGGTGTCTTTG	$(CA)_{15}$	57.4	248	1	-	-	-
Kg040 EF439638	F:CTGCGCAGAAAACAGACCGTCACT R:ACATCCGGACCTGGCCTCCTGTC	$(GT)_{12}$	63.4	150	1	-	-	-
Kg077 EF439639	F:TCTGCCTCTTTCATGTTTTTACTG R:TGTTTGCTATTCTTCTTCCTGTTT	$(GT)_{12}(GA)_4$	57.0	144	1	-	-	-
Kg226 EF439642	F:GAAGCTTGGGTCTTACAGC R:ACACCCCGAGGAGATAACG	$(CA)_{18}$	62.8	287	1	-	-	-
Kg238 EF439643	F:CTGTGCTCATGGGAAAAAGC R:GCAAAGAAATTGGGCATAGG	$(GATG)_4$	56.0	242	1	-	-	-
Kg239 EF439644	F:CAGATGAGTAGCATGTGTGTAAC R:AAGAGTCCAAGAATGAGATAAAAACC	$(CA)_{14}$	62.8	143	1	-	-	-
Kg250 EF439645	F:TTGGCATCTGGTGTAAGTGG R:TGTGGTTGTGAGAGGAGAGG	$(CA)_6$	64.0	213	1	-	-	-
Kg255 EF439646	F:AGGCAGGTAGGCTACAATCG R:GCATGCTTGCATAAGTGTC	$(CA)_9$	58.3	248	1	-	-	-
Kg265 EU285028	F:GGAACCGTTACGTCAGTAGGC R:GTGGAATGTTGTGCTGATGC	$(CA)_{11}$	63.0	229	1	-	-	-
Kg275 EF439647	F:CCAGACTCACACTGGACACG R:GGGACATGAGAGGCAGACC	$(CAGA)_{20}$	56.0	255	5	0.77	0.69	p= 0.4806
Kg286 EF439648	F:CTCATACGCGTGAACAAAGC R:GGAAGCTTGACCCAAAGAGG	$(GGAA)_9$	64.0	219	1	-	-	-
Kg296 EF439649	F:AGCCGACATCGAATGAAAAG R:AAGCTTGGTCACGCAGAG	$(GT)_{11}$	58.3	217	1	-	-	-

Table 3. Allele frequencies for two microsatellite loci estimated from the adult specimens (n = 41); exclusion probabilities calculated under a 'neither parent known' model.

Locus	Exclusion Probabilities	Allele size	Allele frequency
Kg019	0.2477	219	0.354
		221	0.171
		223	0.012
		229	0.415
		231	0.012
		233	0.037
Kg275	0.26311	236	0.462
		240	0.244
		244	0.205
		248	0.051
		252	0.038
Both loci	0.4457		

associated egg masses were consistent with this scenario (Table 4). Our results report an absence of multiple paternity in all egg masses when assessed independently of an associated male. This is consistent with the breeding scenario described by Berra *et al.* (2007). The masses that were captured without any associated males were genotyped to screen for multiple paternity, and we did not find evidence for more than two parents contributing to a mass (Table 5). However, in Table 4, EM-02 is consistent with being sired by AD-02. If AD-02 did sire EM-02, assuming Mendelian inheritance, we would see allele 240 present in the mass. Therefore, we can conclude that AD-02 did not contribute to the mass although the presence of the 236 allele is consistent *or* AD-02 was not the only male to contribute to the mass due to the absence of the 240 allele.

Table 4. Genotypes of egg masses with associated males. Genotype of male is compared to genotypes of multiple embryos in each mass. n = number of eggs/embryos that successfully amplified, having that genotype, at the given locus. *=white egg mass

Male	Locus	Genotype of male	Associated egg mass(es)	Genotypes found in mass	*n*	Consistent with male?
AD-01	Kg019	221/229	EM-01	221/229	5	Yes
				221/221	2	Yes
	Kg275	236/240	EM-01	236/236	3	Yes
				236/240	1	Yes
AD-02	Kg019	219/229	EM-02	219/229	25	Yes
				219/219	10	Yes
				229/229	14	Yes
	Kg275	236/240	EM-02	236/236	28	Yes
				236/244	31	Yes
AD-03	Kg019	219/229	EM-03*	219/229	10	Yes
				221/229	14	Yes
				229/229	9	Yes
				219/221	6	Yes
	Kg275	236/244	EM-03*	236/240	16	Yes
				240/244	25	Yes
AD-04	Kg019	219/229	EM-04	219/229	10	Yes
				221/229	11	Yes
				219/221	13	Yes
				219/219	2	Yes
	Kg275	236/244	EM-04	236/236	25	Yes
				236/244	14	Yes
AD-05	Kg019	229/229	EM-05	219/229	9	Yes
				221/229	10	Yes
	Kg275	244/252	EM-05	228/244	7	Yes
				244/248	6	Yes
				248/252	3	Yes
AD-06	Kg019	219/219	EM-06	219/219	39	Yes
			EM-07	229/229	26	No
				221/229	11	No
	Kg275	240/244	EM-06	236/244	6	Yes
				240/244	5	Yes
				236/248	7	No
				240/248	12	Yes
			EM-07	236/236	13	No
				236/244	14	Yes

The two microsatellite loci are powerful enough to detect multiple paternity according to the PrDM analysis (Table 6). The results indicate that probability increased as the brood size increased, and the probability of detecting multiple paternity increased as the number of sires increased. However, there was still a 58.9 percent chance that multiple paternity can be detected with these markers with the small brood size of 10 when two males contribute to the mass with a skewed ratio (Table 6).

Variation in sequences of control region. DNA sequence data from 780 base pairs of mtDNA control region revealed only four variable sites, defining five distinct haplotypes, among all individuals sampled in northern Australia (Fig. 4). The haplotype of three individuals

Table 5. Egg masses with no associated males. Parental reconstruction performed in Gerud2.0. *=white egg masses

			Embryo genotypic classes						
Egg Mass	Locus	Parent 1 genotype deduced	Class 1	*n*	Class 2	*n*	Class 3	*n*	Parent 2 genotype deduced
EM-08*	Kg019	219/219	219/219	27	219/221	30			219/221
	Kg275	236/236	236/248	33	236/236	25			236/248
EM-09*	Kg019	219/221	219/219	7	219/221	8	219/229	34	219/229
	Kg275	236/236	236/236	15	236/248	3			236/248
EM-10	Kg019	217/221	217/219	2	221/229	46			219/229
	Kg275	236/236	236/236	48					236/236
EM-11	Kg019	221/229	221/229	15	229/229	20			229/229
	Kg275	240/248	240/248	18	248/248	24			248/248
EM-12	Kg019	221/229	221/229	15	229/229	14			229/229
	Kg275	236/244	236/244	21	244/244	15			244/244

Table 6. The values for probability of detecting multiple paternity (PrDM) are shown below the five specific litter sizes tested. Simulations with two and three males were run with equal and skewed mating scenarios.

	Brood size				
Mating scenarios tested	**10**	**30**	**50**	**70**	**90**
2 males (50:50)	0.627	0.736	0.740	0.744	0.739
2 males (66.7:33.3)	0.589	0.732	0.741	0.740	0.742
3 males (33.3:33.3:33.3)	0.809	0.931	0.939	0.940	0.938
3 males (60:25:15)	0.500	0.774	0.840	0.871	0.888

from three Northern Territory river systems disjunct from the Adelaide River were identical to one of the common haplotypes in the Adelaide River population. The four individuals from New Guinea are identical but have on average 24.5 nucleotide substitutions relative to the haplotypes in Australia (Fig. 4). The average p-distance among sampled individuals in northern Australia is extremely low at 0.001. The demographic analyses performed in LAMARC estimated a population growth rate $g = 875.1$, which is consistent with population expansion. The estimation of female effective size (N_{ef}) from the mitochondrial data indicates a range of 11,742–13,252 females. However, only a single theta was used to give an approximate estimate due to the absence of a more accurate mutation rate in D-loop for Kurtidae.

DISCUSSION

Our study is the first to address questions about the life history of *K. gulliveri* utilising genetic markers. *Kurtus gulliveri* is found in very turbid waters where river levels fluctuate up to eight metres daily due to strong tidal surges. They also co-occur with saltwater crocodiles (*Crocodylus porosus* Schneider, 1801), which make field observations of their behaviours dangerous, if not impossible (Berra 2003). However, the nature of the male supraoccipital hook brooding behavior and the structure of the egg masses provide grounds to study this intriguing reproductive strategy as a unique form of paternal care. We tested paternity of specific males with associated egg masses, along with analysis of egg masses collected without associated males, to address questions in the forehead brooding in *K. gulliveri*, and potentially identify patterns that will lead to further studies.

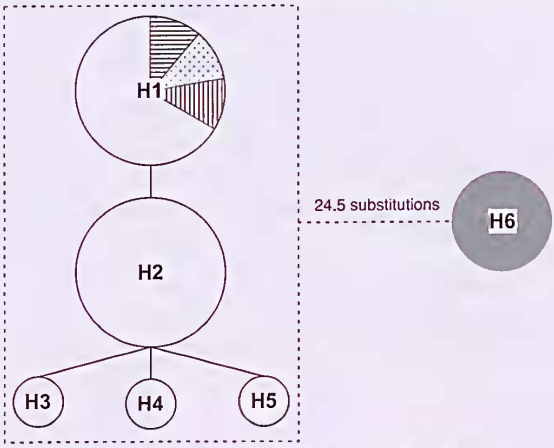

Fig. 4. Parsimony network of six haplotypes (H1–H6) in *Kurtus gulliveri*. Solid lines represent a single substitution. Dotted line marks an average number of 24.5 substitutions from the New Guinean samples to the Australian samples, while the exact connection between them cannot be resolved. Circle sizes are proportional to the number of individuals found for each haplotype (i.e. H1 and H2 were found in 9 individuals, H3 to H5 in one individual and H6 in four individuals). ○ Adelaide River, NT, Australia; ⊜ South Alligator River, NT, Australia; ◯ East Alligator River, NT, Australia; ◍ Daly River, NT, Australia; ● New Guinea.

Microsatellites are excellent markers for paternity studies (Tautz 1989; Goldstein & Schlotterer 1999; DeWoody & Avise 2000) because they are highly variable, codominant, easily amplified, and species-specific. They have proven to be a valuable tool for parentage analyses with as little as two (Kellogg *et al.* 1995) and three variable loci (Zane *et al.* 1999; McCoy *et al.* 2001), and even when few repeat motifs are found using similar techniques (Feldheim *et al.* 2001). However, the extremely low variation among microsatellite markers (with few alleles at high frequencies) found in this study severely limited our ability to provide robust conclusions regarding the hypotheses tested.

The evidence gleaned from the genotypes suggests, but does not prove, that physical proximity during capture means that the male sired or carried the associated mass. One of the males assayed (AD-06, Fig. 2A) produced evidence that rejects the hypothesis of paternity for both masses shown in Fig. 2A. Genotypes at locus Kg275 among the embryos (Table 4) are inconsistent with the genotype of the male. Therefore, close proximity alone is not a sufficient indicator that the male has sired and was carrying the mass. In addition, Male AD-02 (Table 4) is consistent as the sire of EM-02, however this male could not be the only sire contributing to this mass if we assume Mendelian inheritance.

Discovering the mode of fertilisation will greatly augment our knowledge of the mating system of this species. For instance, although it is a common taxon in the rivers where it occurs, it is still unknown how and when a male acquires an egg mass. Berra *et al.* (2007) suggested that the male might spawn by directly pulling the mass from the female, similar to the behaviours seen among apogonid fishes (Vagelli 1999), putative close relatives of Nurseryfish (Johnson 1993; Smith & Wheeler 2006; Thacker 2009). Our samples consist of egg masses associated with putative brooding males (Table 4), in most cases providing consistent evidence to suggest paternity. However, we

cannot reject the idea that males *may* carry unfertilised egg masses. Unfortunately, we cannot ascertain whether the egg mass was unfertilised without direct observation of previtellogenic space and a fertilisation membrane in the field. Failure to amplify microsatellite loci from EM-13 and EM-14 (Table 1) could be due to several factors, among them that the eggs are fertilised but that adequate quantities of DNA could not be recovered. However, recovery of DNA could be hindered by preservation or contamination as well, so the status of these eggs cannot be determined. Since we did not find any egg masses that were homozygous at both loci for all eggs sampled, we cannot definitively say whether any of the white egg masses were actually unfertilised (Table 5).

While addressing these hypotheses, it was surprising to find extremely low genetic variation, given that microsatellites are one of the most highly variable types of genetic markers (DeWoody & Avise 2000). The lack of diversity in the D-loop region – again, one of the more highly variable molecular markers used for population studies – is consistent with this observation which has also been documented in populations of *Esox lucius* (Jacobsen *et al*, 2005). The estimated female effective population size (approx. 12,000) for *K. gulliveri* is comparable to that of a genetically depauperate population killifish, *Fundulus sciadicus* Cope, 1865 in streams of Nebraska (N_{ef} approx. 7000) (Li *et al.* 2009), and significantly less than a highly abundant and fecund characiform, *Prochilodus nigricaus* Stix & Agassiz, 1829 (N_{ef} approx. 600,000) from a single South American drainage (Orti *et al.* unpublished data). It is often acknowledged that freshwater fishes exhibit lower genetic variation and increased population structure in comparison to marine fishes (e.g. Ward *et al.* 1994), which was reinforced by the genetic data observed in the Nurseryfish. However, individuals sampled from disjunct drainages in Australia did not show any divergence from each other while samples from New Guinea were very different from the Australian fish. In addition, the genotypes of the New Guinea specimens revealed novel genotypes not found in the Australian populations (data not shown). A possible explanation for this phenomenon could be historical bottleneck and range expansion events caused by Late Pleistocene sea level fluctuations (Voris 2000), but these all are only speculative given the limited power of the current data set. More samples from the entire range are necessary to test this hypothesis. Other variable markers such as amplified fragment length polymorphism (AFLPs) and single-nucleotide polymorphism (SNPs) may be needed to address the unanswered questions concerning historical demography and the therefore clandestine mating system in these fascinating fishes.

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